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Genetic Analysis of Bristle Loss in Hybrids between *Drosophila melanogaster* and *D. simulans* Provides Evidence for Divergence of *cis*-Regulatory Sequences in the *achaete–scute* Gene Complex

Nick Skaer and Pat Simpson

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, BP 163, 67404 Illkirch Cedex, C.U. de Strasbourg, France

The two closely related species of *Drosophila, D. melanogaster* and *D. simulans,* display an identical bristle pattern on the notum, but hybrids between the two are lacking a variable number of bristles. We show that the loss is temperature-dependent and provide evidence for two periods of temperature sensitivity. A first period of heat sensitivity occurs during larval development and corresponds to the time when the prepattern of expression of genes whose products activate *achaete–scute* in the proneural clusters preceding bristle precursor formation is established. A second period of cold sensitivity corresponds to the time of emergence of the bristle precursor cells and the maintenance of their neural fate, a process requiring high levels of Achaete–Scute. Expression of *achaete–scute* at these two critical periods depends on *cis*-regulatory elements of the *achaete–scute* complex (AS-C). The differences between males, which have only one copy of the X-linked AS-C from *D. simulans,* and females, which have copies from both parental species, are compared, together with the effects of crossing in different rearrangements of the *D. melanogaster* AS-C that delete regulatory and/or coding sequences. We provide evidence that bristle loss in the hybrids may result from a decrease in the level of transcription at the AS-C and argue that interaction between *trans*-acting factors and *cis*-regulatory elements within the AS-C has diverged between the two species.

Key Words: Drosophila; **hybrids; sensory organs;** ***achaete–scute;*** **regulatory sequences; evolution.**

INTRODUCTION

The pattern of large sensory bristles on the notum of *Drosophila melanogaster* is stereotyped and rarely varies between different individuals. It is widespread throughout most of the 2000 or so species of the family Drosophilidae and is also extremely old: specimens preserved in amber with almost identical patterns have been described that date from up to 40 million years ago (Grimaldi, 1987, 1990). The positions of these bristles are likely to be important for the fly's behavior and indeed the neuronal specificity of the bristle organ is dependent upon the site within the epithelium at which the bristle precursor cell arises (Ghysen, 1980). In other families of Schizophoran Diptera, however, the arrangement of bristles, while often equally stereotyped, is different and some of these patterns, too, are phylogenetically old (McAlpine, 1981). It is not known how the different patterns in different species are constructed or how they are maintained over long periods of evolutionary time.

The genetic control of bristle patterning in *D. melanogaster,* however, has been extensively studied. The genes of the *achaete–scute* complex (AS-C) encode related basic helix–loop–helix (bHLH) proteins characteristic of a family of transcriptional regulators that work as heterodimers together with the product of the gene *daughterless* (Ghysen and Dambly-Chaudière, 1988; Cabrera and Alonso, 1991; Villares and Cabrera, 1987; Gonzalez *et al.,* 1989). Expression of these genes provides cells with neural potential, allowing them to develop into nerve cells. In the thoracic imaginal disc the two genes *achaete* (*ac*) and *scute* (*sc*) are expressed in small clusters of cells called proneural clusters that prefigure the sites of each of the future bristles (Romani *et al.,* 1989; Cubas *et al.,* 1991; Skeath and Carroll, 1991). Expression is then progressively refined to single, spaced precursor cells by means of a mechanism of lateral

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signalling that is mediated by the Notch signaling pathway (Artavanis-Tsakonas *et al.,* 1995; Kimble and Simpson, 1997). The *ac-sc* genes share *cis*-regulatory enhancer sequences that are scattered over about 100 kb of DNA (Ruiz-Gomez and Modolell, 1987; Gomez-Skarmeta *et al.,* 1995). The *cis*-acting elements respond to local positional cues, conveyed by *trans*-acting factors that regulate the dynamic spatial and temporal expression patterns of these genes.

Some of the upstream transcriptional activators of *ac* and *sc* are known; they are expressed in distinct domains over the epithelium of the notal disc and are thought to define a "prepattern" (Stern, 1954; Simpson, 1996; Modolell and Campuzano, 1998). Four activators, Araucan, Caupolican and Mirror in the *iroquois* complex and Pannier, are required for the bristles of the lateral and medial halves of the notum, respectively (Gomez-Skarmeta *et al.,* 1996; Leyns *et al.,* 1996; Ramain *et al.,* 1993; Cubadda *et al.,* 1997; Haenlin *et al.,* 1997; Kehl *et al.,* 1998). Pannier has been shown to act through the dorsocentral *cis*-regulatory element to control development of the two dorsocentral bristles, but is negatively regulated by the product of the *u-shaped* gene. Together the partially overlapping but distinct expression domains of *pannier* and *u-shaped* lead to the precise positioning of the dorsocentral bristles (Cubadda *et al.,* 1997; Haenlin *et al.,* 1997; Garcia-Garcia *et al.,* 1999).

The two species of *Drosophila, D. melanogaster* and *D. simulans,* are closely related and are separated by about 0.8 to 3 Myr (Lemeunier *et al.,* 1984). They display only slight differences in genome structure and morphology (Lemeunier *et al.,* 1984). The notal bristle pattern is indistinguishable between the two. Crosses between these species are possible and give rise to semiviable, but mostly sterile, progeny (Sturtevant, 1919, 1920; Lemeunier *et al.,* 1984; Davis *et al.,* 1996; Hutter, 1997). Strikingly, the hybrids are lacking a variable number of bristles on the thorax (Sturtevant, 1920, 1929; Biddle, 1932). Earlier authors noted that the bristle loss appears to be nonspecific and is sensitive to a number of factors, including the genetic background (Sturtevant, 1920, 1929; Biddle, 1932; Kerkis, 1933; Watanabe *et al.,* 1977; Takano, 1998a). Here we have investigated the developmental basis for the bristle loss. We find that under rigorously controlled, uniform conditions, the bristles are reproducibly lost in a specific order. We find that this loss is temperature-dependent and provide evidence for two periods of temperature sensitivity. A first period of heat sensitivity is situated during larval development and corresponds to the time when the prepattern and the proneural clusters are being established in the thoracic disc. A second period of cold sensitivity corresponds precisely with the time of emergence of the bristle precursor cells. The differences between males, which have only a copy of the X-linked AS-C from *D. simulans,* and females, which have copies from both parental species, were compared with the effects of crossing in different rearrangements of the AS-C, as well as mutants for *pannier* and *iroquois* from the *D. melanogaster* parent. From these results we argue that the interaction between *trans*-acting factors and the *cis*-regulatory elements within the AS-C has changed between the two species.

MATERIALS AND METHODS

Fly Crosses and Strains

Most crosses were performed with an inbred strain of *D. melanogaster,* Ore-R, that had been maintained in our laboratory for many years, and a strain (S12a) of *D. simulans* obtained from the Umea stock centre. Crosses between *D. melanogaster* females and *D. simulans* males give only female progeny; the males are not viable (Sturtevant, 1919, 1920; Lemeunier *et al.,* 1984; Hutter, 1997). Such females carry one X chromosome (with the AS-C) from each parent species. Hybrid males carrying the *D. simulans* X chromosome can be obtained from the reciprocal cross between *D. simulans* females and *D. melanogaster* males. This cross is difficult to perform and so we also obtained such males from a cross between a *D. melanogaster* compound X stock and *D. simulans* males. In order to minimize phenotypic variability in the progeny of these crosses, the following protocol was used throughout. Crosses were conducted between virgin female *D. melanogaster* and male *D. simulans* flies collected over a period of no longer than 3 days and grown under controlled temperature conditions. In most instances four simultaneous replicas of each cross were set up, two containing 25 females and 25 males, the other two each containing 50 females and 50 males. Parental flies were maintained at the temperature at which the experimental cross was carried out. At 25°C transfer of parental flies was effected every 4 days and at 18°C every 7 days. Wherever possible, for each replica, only the first vial containing hybrids was sampled, from which only the first 25 progeny to eclose were scored for bristle loss on both heminota (therefore, for each cross $4 \times 25 \times 2 = 200$ heminota were scored).

The following mutant strains of *D. melanogaster* were employed: *-Df(1)sc^{B57} w s/FM7, -Df(1)260-1 w s/FM7c, -In(1)ac^{3} w s/FM7c, -In(1)ac^{3} sc$^{10\text{-}1}$ w s/FM7c, -y; pr cn Dp(1;2)sc^{19}/CyO, -HSSC-2* (described by Rodriguez *et al.,* 1990), *-Df(1)sc^{2} w s/FM7c, Df(1)sc^{6} w s/FM7c, -Df(3L)iro^{2} Sb^{2}/TM3, Sb Ser, -pnr^{VX6} kar^{2} ry^{506}/TM3 Sb Ser, -Df(1)sc^{B57} w s/FM7; pnr^{VX6} kar^{2} ry^{506}/TM3 Sb, -Compound-1 DX y f/Y, -Compound-1 DX y w f/Y, -sc^{M6}/FM7* (Gomez-Skarmeta *et al.,* 1995). For description of the mutations and rearrangements see Lindsley and Zimm (1992).

Balancer Chromosome Controls

Balancer chromosomes were evaluated for their effects on bristle loss (and thus their suitability as internal controls) by crossing *D. melanogaster* females carrying one copy of the Balancer plus the corresponding wild-type chromosome to *D. simulans* males and comparing the two classes of sibling hybrid progeny for bristle loss. It was found that first chromosome Balancers (FM7, FM7c) had no significant effect on bristle loss and that the second chromosome Balancer (CyO) caused slight, but not statistically significant, differences for some bristles when compared to hybrids carrying the wild-type chromosome. Hybrids carrying third chromosome Balancers were not viable at sufficiently high frequencies for statistical analysis to be performed. Consequently, those hybrids

bearing the *pannier* and *iroquois* deletions have no internal controls, and these results have been compared to hybrids from the wild-type cross conducted at the appropriate temperature.

Temperature-Sensitive Periods

Females. Ore-R *D. melanogaster* females were crossed to *D. simulans* males, and the egg lays of newly mated females were synchronized. Eleven groups of 50 females were left to lay in culture tubes containing fresh yeast for a period of 2 h at 25°C. The females were subsequently transferred to fresh tubes every 8 h an additional three times giving a total of 44 synchronized cultures. The same procedure was carried out at 18°C with the difference that 12 groups of females were set up giving a final total of 48 synchronized cultures. After 12 h, and every subsequent 12 h, four cultures were transferred from 25 to 18°C. The same protocol was followed for the reverse shift, from 18 to 25°C, but cultures were transferred every 24 h after an intial 36-h period. For each shift a minimum of 200 heminota were scored.

Cold pulses were also administered to cultures that were synchronized relative to the point of pupariation. Ore-R *D. melanogaster* females were crossed to *D. simulans* males and hybrids were raised at 25°C until the beginning of pupariation. White prepupae were collected and divided into two groups. One group was transferred to 18°C and left to develop for a period of 24 h before being returned to 25°C. Of the other group, half were shifted to 18°C 24 h after pupariation formation (APF), while the other half were left to continue development at 25°C.

Males. Compound X *D. melanogaster* females were mated with *D. simulans* males. Seven groups of 30 females raised at 18°C were crossed to 30 males. After 1 week, the females were separated and isolated at 18°C in small tubes containing fresh yeast for a period of 8 h. The females were then transferred a further three times giving a total of 28 synchronized cultures which were shifted to 25°C at 48-h intervals up to 336 h timed from the end of the laying period. The F1 hybrid progeny were collected over a 3-day period beginning when the first fly eclosed. The same procedure was carried out at 25°C except that females were raised at 25°C and tubes were shifted to 18°C at 24-h intervals up to 168 h.

Bristle Precursor Analysis

To generate female hybrids *D. melanogaster* females of the genotype $neu^{A101}(lac, ry^{+})\ kar^{2}\ ry^{506}/TM6C\ ry^{CB}\ Sb\ Tb$, which carry a *lac-Z* reporter construct under control of the *neuralized* promoter (Huang *et al.*, 1991), were crossed to *D. simulans* males according to the protocol previously described and cultures were raised at 18°C. To generate male hybrids compound X *D. melanogaster* females XXY; $neu^{A101(lac,\ ry+)}\ kar^{2}\ ry^{506}/TM6C\ ry^{CB}\ Sb\ Tb$ were crossed to *D. simulans* males. Larvae and pupae carrying the *lac-Z* insertion were selected by discriminating against the *Tb* marker. Prepupal discs were dissected from white prepupae in standard PBS and then stained for *lac-Z* expression using an antibody to *β-Galactosidase* according to standard protocols. For pupal nota, white prepupae were collected and kept at 18°C for an additional 24 or 32 h. Pupal nota were then dissected out under standard PBS and stained following the same protocol as for the discs.

Duration of Development

The duration of development was measured in *D. melanogaster* and *D. simulans* and in hybrids of the cross between *D. melanogaster* females and *D. simulans* males. Owing to the tendency of adults to hatch preferentially at dawn it was decided that the period from egg laying to pupariation would give a more accurate comparison of developmental durations and at the same time reflect the developmental stages relevant to bristle development. Egg lays of newly mated females were synchronized, and four groups of 50 females were left to lay on nutrient agar plates containing fresh yeast for 1 h at 25°C. After 36 h at 25°C 50 larvae from each plate were removed and transferred to a fresh culture tube. These tubes were subsequently checked at regular periods for pupae, and the average pupariation time was calculated for each culture.

Heat Shock Scute

Homozygous *D. melanogaster* females carrying an extra copy of the *scute* gene under the control of the *hsp70* promoter (*HSSC-2-2*, Rodriguez *et al.*, 1990) were crossed to *D. simulans* males, and hybrids were raised at 18°C. When pupae appeared in the tubes, cultures were transferred to 37°C for 3 h before being returned to 18°C. White prepupae were collected at regular intervals both before and after heat shock, and the stage of development at which the heat shock had taken place was calculated retrospectively. The same protocol was carried out on hybrids derived from the wild-type cross to control for effects of the temperature change.

Statistical Analysis

All data are quoted and graphically represented plus or minus the mean standard error. Data from the independent cultures were compared using a Student's *t* test. Where internal controls were available tests of significance were performed using a paired, two-tailed, *t* test. In instances where internal controls were not available a homoscedastic, two-tailed *t* test was used. Results were considered significant when a *P* value of 0.05 or less was returned.

RESULTS

Duration of Development in D. melanogaster and D. simulans

The time taken between egg laying and pupariation was measured for the two species *D. melanogaster* and *D. simulans* (hereafter referred to as simply *melanogaster* or *simulans*). We found that, at 25°C, *simulans* takes significantly longer to develop than *melanogaster:* the time between egg laying and pupariation was 156 ±1.18 h and 137 ± 3.45 h, respectively ($P < 0.01$). The female hybrids resulting from a cross between *melanogaster* females and *simulans* males display a developmental time between the two parental species (138 ± 3.37 h).

In the Hybrids Specific Bristles Are Lost with Reproducible Frequency

There are 11 large bristles or macrochaetes present on each heminotum of both species (Fig. 1). The hybrids

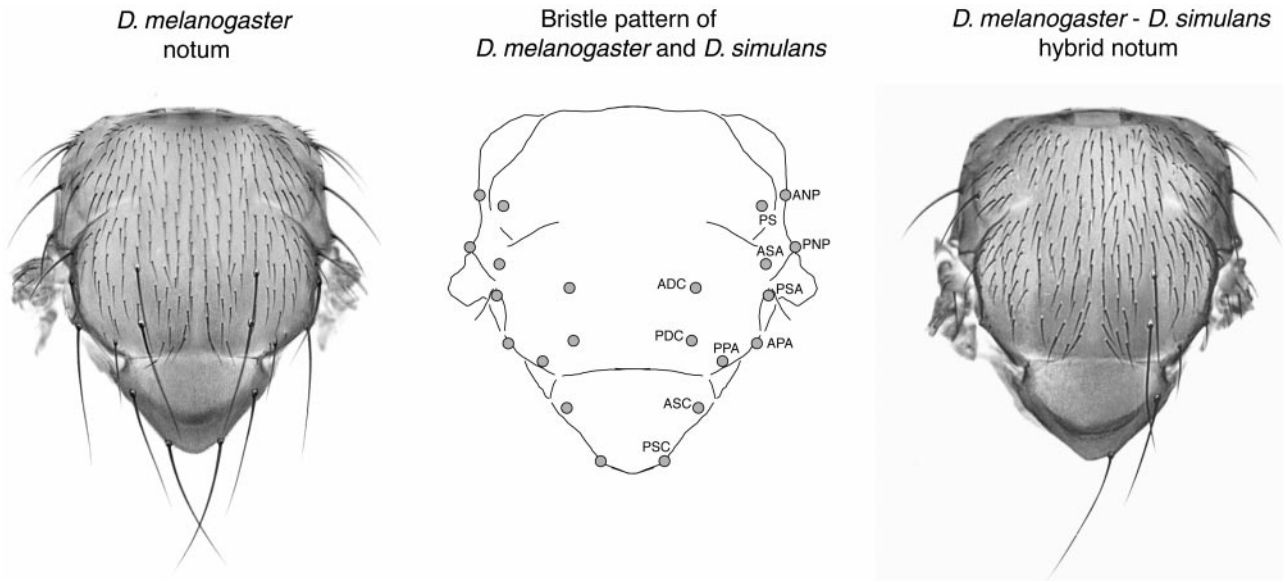


FIG. 1. The bristle pattern on the dorsal notum of *D. melanogaster, D. simulans,* and a *melanogaster*/*simulans* hybrid. A wild-type notum of *D. melanogaster* is shown, together with a hybrid thorax in which a number of bristles can be seen to be missing. The two parental species display an identical bristle pattern of 11 macrochaetes the names of which are abbreviated as follows: ANP, anterior notopleural; PNP, posterior notopleural; PS, presutural; ASA, anterior supraalar, PSA, posterior supraalar; APA, anterior postalar; PPA, posterior postalar; ADC, anterior dorsocentral; PDC, posterior dorsocentral; ASC, anterior scutellar; PSC, posterior scutellar.

display a variety of bristle abnormalities, including missing macro- and microchaetes, duplicated bristles, and occasional bristles with morphological defects such as malformed shafts and duplication of shafts and/or sockets. Bristle loss is by far the most common defect (Fig. 1). Like earlier authors we found that bristle loss is sensitive to both genetic and environmental factors such as parental strain differences, age of parents, age of culture medium, temperature, crowding of cultures, and light or dark. We therefore derived conditions under which all of these factors were maintained as constant as possible. The effects of intraspecific variation have been recently explored by Takano (1998a); to minimize such effects we have collected all data (other than crosses involving *melanogaster* mutant strains) from the same two inbred strains of *melanogaster* and *simulans*. In addition we have employed the rigorous protocol described under Materials and Methods.

Under these conditions, at 25°C, bristle loss is not stochastic and the frequency of loss of specific bristles is reproducibly constant (Fig. 2). Throughout the text we use the common abbreviations for bristle names as given in the legend to Fig. 1. The sensitivity of each bristle to loss differs reproducibly between male and female hybrids. In female hybrids, the PSA is by far the most frequently lost, followed by the ASA and APA and then by the ADC, PNP, ANP, PS, PDC, PPA, ASC, and PSC. In male hybrids this order is partially reversed for a number of bristles, the ASC and PSC being among the most sensitive.

Bristle Loss Is Temperature-Dependent

As noted previously, one strong variable affecting bristle loss is temperature (Kerkis, 1933; Watanabe *et al.*, 1977). In female hybrids at 18°C, most bristles are lost at a much greater frequency than at 25°C (Fig. 2). Indeed at this temperature the differences between specific bristles, observed at 25°C, is not apparent. Nine of the 11 macrochaetes were seen to be strongly cold-sensitive in female hybrids. One bristle, the PSA was heat-sensitive, being lost more frequently at 25°C than at 18°C. The ASA did not appear to be temperature-dependent. These latter bristles are, notably, the most sensitive to loss in female hybrids. We also looked at crosses performed at 30 and 16°C and obtained similar, very slightly more extreme, results (not shown). In contrast, in males the bristles are all heat-sensitive and are lost at a greater frequency at 25°C than at 18°C (Fig. 2).

The Cold-Sensitive Periods Correlate with the Time of Precursor Formation

To characterize the temperature dependence further, a series of temperature shift assays was performed. Animals were grown at 18°C and shifted to 25°C at specific times throughout development and vice versa (as described under Materials and Methods). Loss of bristles in male progeny was not found to be cold-sensitive (Fig. 3B). In contrast, a specific cold-sensitive period could be defined for the bristle

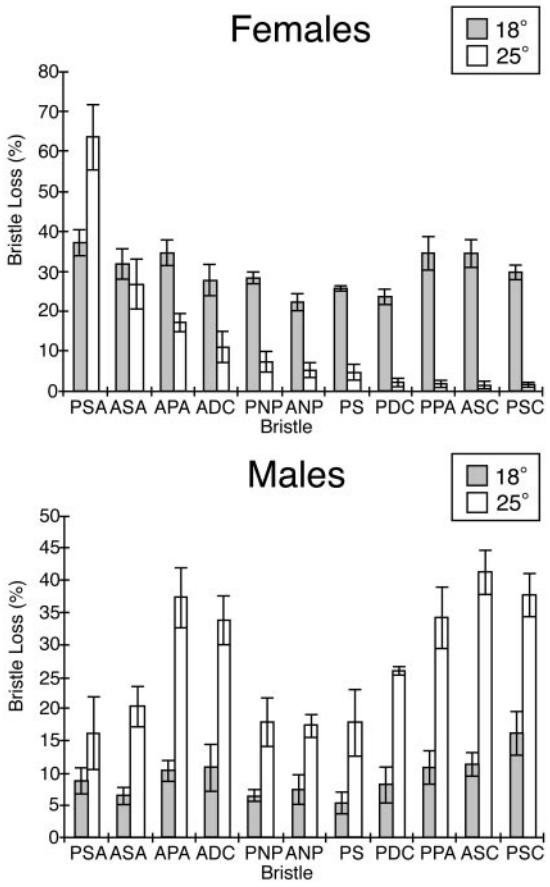


FIG. 2. The frequency of loss of bristles on the dorsal notum of male and female *melanogaster*/*simulans* hybrids. The loss of each bristle is given as a percentage from the 200 or more heminota that were scored for each cross (see Materials and Methods). Females were obtained from a cross between *melanogaster* females and *simulans* males. Males were obtained from a cross between *melanogaster* compound X (XXY) females and *simulans* males. The results are given for flies grown at 18 (filled bars) or 25°C (open bars). It can be seen that, in females, all bristles, except the PSA and ASA, are lost at greater frequency at 18°C than at 25°C ($P < 0.05$). In males, all bristles except the PSA and PS are lost at greater frequency at 25°C than at 18°C ($P < 0.05$ for all bristles except the PSA ($P < 0.26$) and PS ($P < 0.059$)). For animals of either sex grown at 25°C, specific bristles are lost at a characteristic frequency. These differences are not apparent at 18°C. Note that the scale on the *Y* axis of the two histograms is different.

loss in female progeny: when the results for all bristles are pooled, a broad period of sensitivity to cold is defined that falls between 84 and 132 h in the 25 to 18°C shifts and between 156 and 252 h in the 18 to 25°C shifts (the cold-sensitive period, CSP; see Fig. 3A). This period corresponds to the mid-third larval instar to early pupal stages. This is the time at which the bristle precursor cells are born. In *melanogaster* each precursor has been shown to arise within a particular time window that may be long or short (Fig. 4; Romani *et al.*, 1989; Huang *et al.*, 1991; Cubas *et al.*, 1991; Skeath and Carroll, 1991). Examination of individual bristles provides a more detailed picture in which it can be seen that there is a fairly good correlation between the CSPs and the time of birth of the respective precursors (see Fig. 4). Bristles, such as the PDC, whose precursor forms early and over an extended time window, have a correspondingly early and broad CSP. Conversely, bristles such as the PPA, whose precursor arises late and from a narrow time window, display much sharper and narrower CSPs that are correspondingly late. The CSPs for the eight strongly cold-sensitive bristles are shown in Fig. 4 and are roughly consistent in each case with the time of precursor formation.

Although the CSPs for these eight bristles are approximately correlated with the time of precursor formation, a fine resolution is limited by the natural variation in developmental rate of the cultures and the frequency of the shifts. A more refined analysis is possible by administering brief cold pulses at specific developmental times in precisely synchronized cultures. Puparium formation provides a reliable marker with which to stage the animals, particularly since the bristle precursors fall naturally into two groups, those formed before and those formed after this point (see Fig. 4; Huang *et al.*, 1991). Cold pulses (18°C) were administered for a period of 24 h starting at puparium formation to cultures that were otherwise grown at 25°C. Some cultures were also shifted from 25 to 18°C 24 h APF, when all bristle precursors have been formed. This latter treatment had no effect on any of the bristles, compared to cultures maintained at 25°C (not shown). However, hybrids subjected to a cold pulse for 24 h shortly after puparium formation displayed significantly higher loss of the PPA, PS, and ADC bristles than those left at 25°C (Fig. 4). Precursors for the PPA, PS, and ASA are the only ones to arise after pupariation (Huang *et al.*, 1991; for the ASA see below). The ADC precursor arises shortly before puparium formation. There are less significant, but nonetheless noticeable, increases in the frequency of loss of the ANP and ASC which form just prior to the ADC. The PNP, PDC, and PSC, whose precursors form earlier, some time before puparium formation, show no significant differences from the controls (Fig. 4). There is thus a strong correlation for loss of the cold-sensitive bristles in female hybrids, between time of exposure to low temperature and the period at which the precursors form in *D. melanogaster*. The CSP appears to be situated at, or shortly after, precursor formation.

The Heat-Sensitive Period Correlates with Establishment of the Prepattern for the Proneural Clusters

In the female hybrids three bristles behaved differently from those described above. The PSA, the only clearly

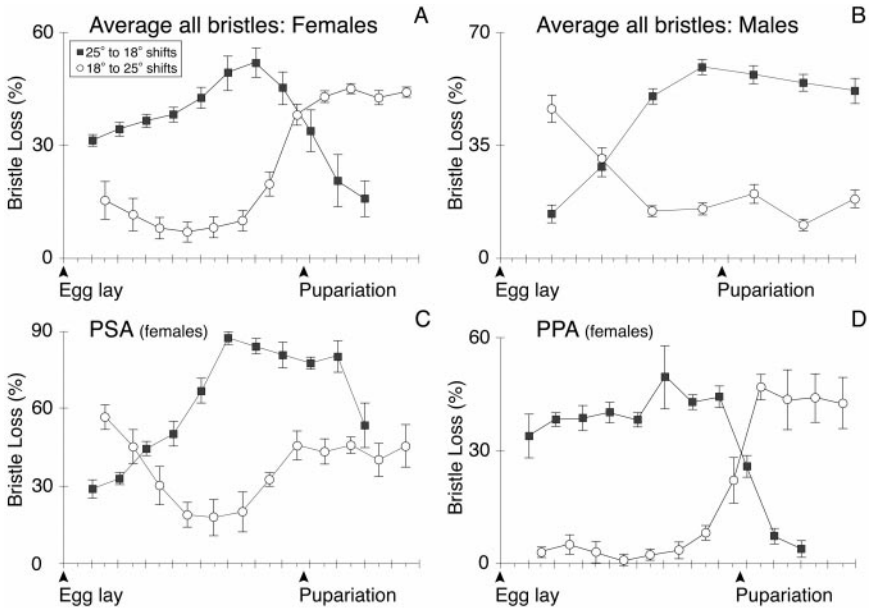
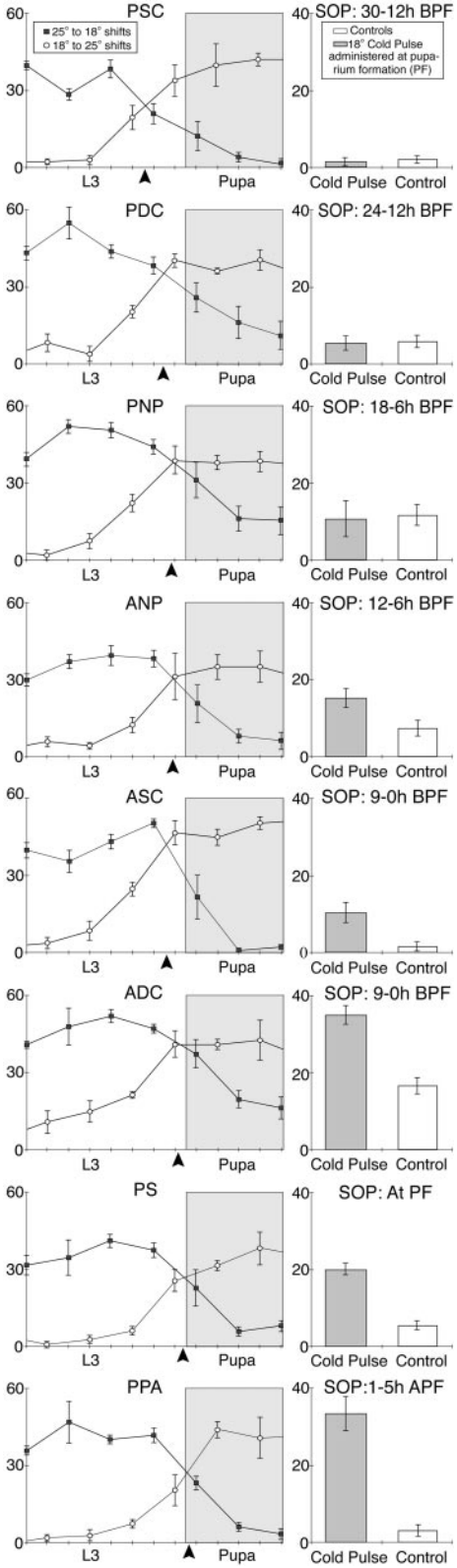


FIG. 3. The heat-sensitive periods (HSP) for bristle loss in male and female *melanogaster*/*simulans* hybrids. For females the results for two individual bristles, the PSA (C) and PPA (D), are shown, as well as the average for all bristles pooled together (A). The curves correspond to batches of animals grown either at 18°C and shifted up to 25°C at specific intervals (circles) or at 25°C and shifted down to 18°C at specific intervals (squares) throughout development. Arrowheads at the bottom indicate the time of egg laying and the approximate time of pupariation. The loss of each bristle is given as a percentage from the 200 or more heminota that were scored for each shift (see Materials and Methods). In males (B) the crossover point during early larval development indicates the HSP. In females the late crossover point indicates the late cold-sensitive period (CSP), but note the fall in the 18°C curve before the rapid rise at the onset of the CSP and the corresponding rise in the 25 to 18°C curve before the rapid fall when the CSP begins. This indicates some sensitivity to heat during early larval stages. This is in contrast to the curves for the PPA which are level before rising and falling at the CSP. Note that the PSA appears to have two crossover points, one corresponding to an early HSP and a weaker one to a late CSP. Note that the scales on the *Y* axes are not the same for each graph.

heat-sensitive bristle in females (Fig. 2), shows an early, broad period of sensitivity to high temperature during larval development, long before the precursor for this bristle appears at 6–12 h before puparium formation (BPF; heat-sensitive period, HSP; Fig. 3C). Interestingly, this bristle also displays a mild CSP much later in development at the time of precursor formation. The ASA bristle also appears to have two periods of temperature sensitivity, an early HSP and a later CSP (not shown). Thus the two TSPs cancel out for this bristle and explain the apparent lack of temperature sensitivity seen in crosses grown continuously at 18 or 25°C (Fig. 2). The APA similarly displays a mild, late CSP but is not clearly heat-sensitive (not shown). However, the shape of the curve corresponding to the 25 to 18°C shift for the APA and some other bristles deserves comment. In a classical TSP profile, the curves corresponding to both the 18 to 25 and the 25 to 18°C shifts are initially straight and level before curving upward and downward, respectively (for example, see the PPA in Fig. 3D). In the case of the APA and, to a lesser extent, the PS, ADC, ANP, PNP, and PDC, the 25 to 18°C shifts display curves that rise steadily for some time before falling rapidly when the CSP begins (not shown). This can be clearly seen when the data from all bristles is pooled (Fig. 3A). This may represent a slight heat sensitivity of these bristles during larval development, such that the longer the time spent at 25°C during this period the greater the bristle loss. For at least two bristles, the PNP and the ADC, a corresponding slight fall in the 18 to 25°C curve, before the rapid rise at the onset of the CSP, was also apparent (not shown). This too is visible for the average of all bristles (Fig. 3A). These results suggest that there are two TSPs in female hybrids, an early heat-sensitive one and a late cold-sensitive one.

In male hybrids no bristles are cold-sensitive; instead they are all heat-sensitive (Fig. 3B). A broad period of heat sensitivity was defined that spanned early larval development. This is the period during which the prepattern of gene expression required to initiate expression of *ac-sc* in the proneural clusters (PNC) is established. The cross used for determination of the TSP in males (see Materials and



Methods) was difficult to perform, generated fewer progeny and therefore less precise timing, and so we did not examine each bristle individually.

Bristle Precursors Are Missing by Early Pupal Stages

Since the CSP is correlated with the time of emergence of the bristle precursors, we looked to see whether the precursors form properly in the hybrids using the enhancer trap line A101, a *lacZ* insertion in the *neuralized* gene that labels all bristle precursors early in their formation (Huang *et al.*, 1991; Boulianne *et al.*, 1991). We examined wild-type *melanogaster* and hybrid thoracic discs from white prepupae and pupae (at 24 and 32 h APF) grown at 18°C (Fig. 5). In both male and female white prepupae, precursors known to form before puparium formation in *melanogaster*, were found to be present in most discs. Rarely, a precursor was seen to be missing. We noted two missing precursors in a study of 50 imaginal discs from female larvae raised at 25°C (i.e., 0.4% of precursors) and 4 missing precursors in a study of seven imaginal discs from male heat-pulsed larvae (5.2%). Figure 5B shows an example in which the PNP precursor is absent. This precursor forms early, 18–6 h BPF (Huang *et al.*, 1991). At 24 h APF, however, many of the precursors cannot be seen with the A101 label (cf. Figs. 5C–5F). This is even more pronounced at 32 h (A101 is expressed up until the adult stage in wild-type animals). Occasionally, a faint trace of label is present at the site where a precursor would normally be found, suggesting that the precursor cells may not die but rather lose their neural fate. This is illustrated in Fig. 5F, where two small patches of label can be seen at the site of the ASC, giving the

FIG. 4. Cold-sensitive periods (CSP) for the loss of eight bristles in female *melanogaster*/*simulans* hybrids. The curves correspond to batches of animals grown at 18°C and shifted up to 25°C at specific intervals (circles), or grown at 25° and shifted down to 18° at specific intervals (squares) during development. The stage of development is shown at the bottom, where L3 indicates the third larval instar and the shading indicates the early pupal period. The loss of each bristle is given on the left, as a percentage from the 200 or more heminota that were scored for each shift (see Materials and Methods). Results for the eight bristles are given in the order in which their precursors are known to arise in *D. melanogaster*, shown on the right-hand panel, SOP (sensory organ precursor) in hours before (BPF) or after (APF) puparium formation. It can be seen that the CSP is situated later in later born precursors: the crossover point of the two curves (indicated by arrowheads) is progressively shifted toward the right in the graphs. The effects of 24 h 18°C cold pulses, administered at pupariation, to animals grown at 25°C, are shown on the right. The loss of bristles whose precursors are known to form late is increased ($P \leq 0.01$ for the PPA, PS, and ADC; $P < 0.1$ for the ASC and ANP; $P > 0.1$ for the PNP, PDC, and PSC).

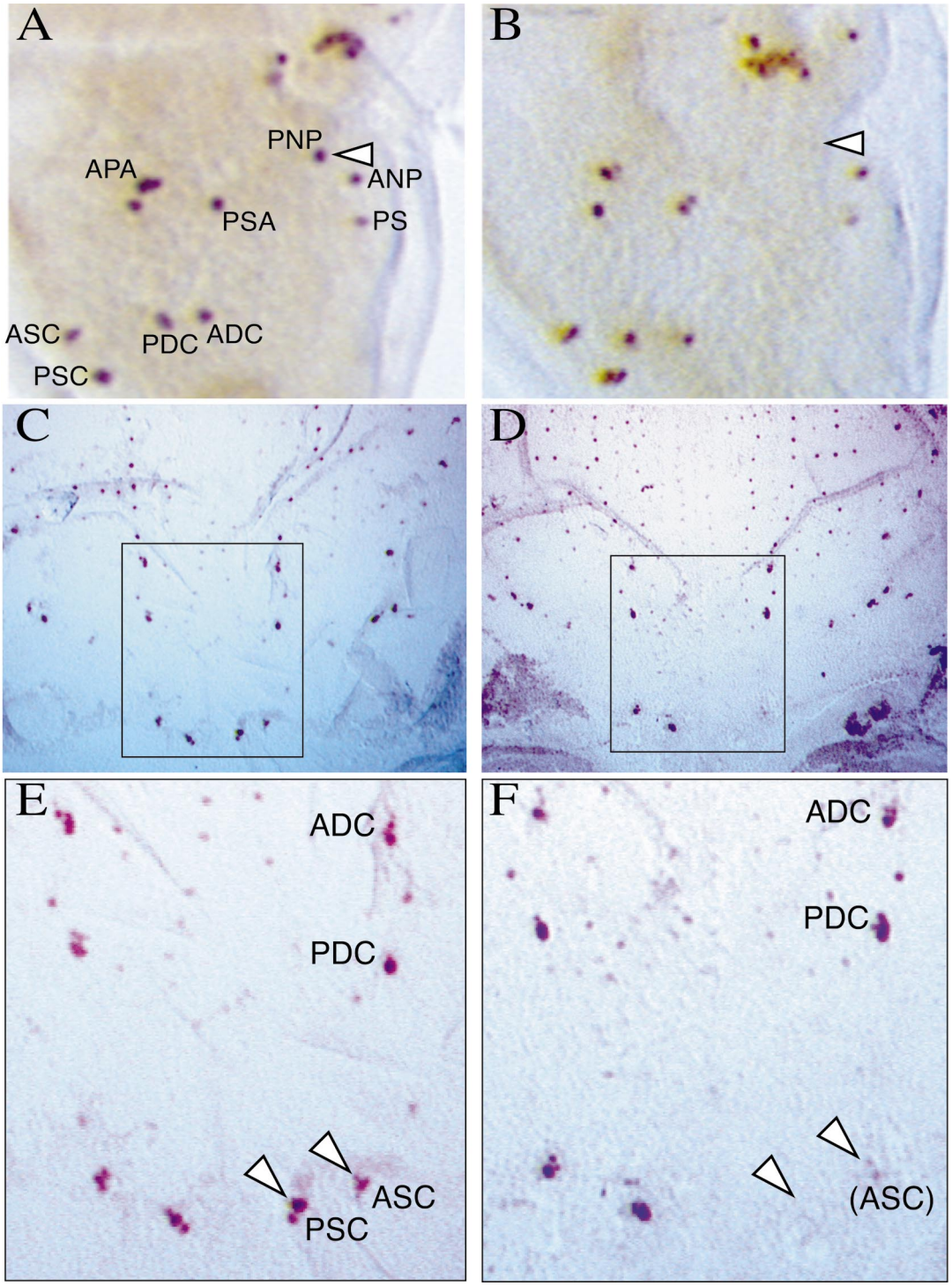


FIG. 5. Analysis of sensory organ precursors in the prepupal and early pupal imaginal discs of female *melanogaster*/*simulans* hybrids. (A, C, and E) *neu*-A101 staining of the precursors in wild-type *D. melanogaster* disks at white prepupal (A) and 24 h pupal stages (C, enlarged in E). The precursors are labeled as in the legend to Fig. 1. (B, D, and the enlargement in F) Similar stages from hybrid *melanogaster*/*simulans* disks. In (B), the PNP precursor is missing and in (D) and (F) precursors for the ASC and PSC are absent (arrowheads). Note that, in (F), at the site where the ASC should be located, faint labeling can be seen in two patches that are considerably smaller than the precursors or their progeny at other sites.

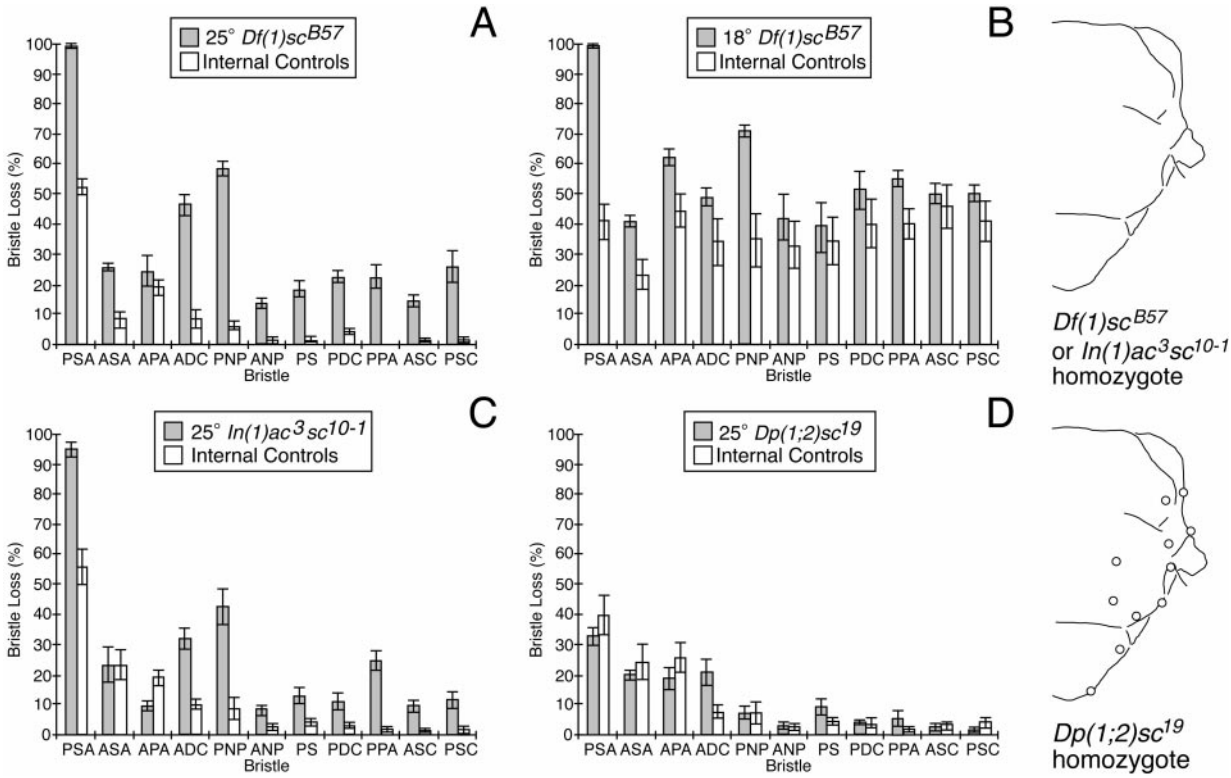


FIG. 6. Frequency of bristle loss in female *melanogaster*/*simulans* hybrids heterozygous for a deletion, duplication, or rearrangement of the *achaete–scute* complex inherited from the *melanogaster* parent. The loss of each bristle is given as a percentage from the 200 or more heminota that were scored for each cross (see Materials and Methods). Sibling flies carrying the *melanogaster* Balancer chromosome were used as controls (see Materials and Methods). Results for $Df(1)sc^{B57}$ are shown at 25 (A) and 18°C (B). Note that at 25°C, with the exception of the APA, the frequency of loss, relative to control siblings is greatly accentuated ($P < 0.05$ for all bristles except the APA ($P = 0.13$)). At 18°C the difference between experimental and control siblings is much less marked and is not significant for a number of bristles. Flies inheriting $Dp(1;2)sc^{19}$ (D) do not display a reduced frequency of bristle loss at 25°C compared to control siblings. The slightly increased loss of the ADC remains unexplained. Results for $In(1)ac^{3}\ sc^{10\text{-}1}$ at 25°C (C) show that with the exception of the ASA and APA, bristles are lost at a higher frequency than controls, but the effect is not as severe as that seen for $Df(1)sc^{B57}$ ($P < 0.05$ for all bristles except the PS ($P = 0.12$), ASC ($P = 0.073$), and PSC ($P = 0.14$) whose loss is not statistically significant). The slight rescue of the APA bristle remains unexplained. The phenotype of *melanogaster* flies (or tissue patches in mosaics) homozygous for these mutations is indicated on the right.

impression that the labeled cell has shrunk to the size of surrounding epidermal cells. The results suggest that many of the precursors first form and then later regress. Similar results were described by Takano (1998a). Since β-galactosidase is known to perdure for as long as 18–24 h within a cell after its synthesis has stopped (Morrison *et al.*, 1999), loss of the precursor cell fate probably starts some time shortly after their formation.

An Exogenous Supply of Scute Delivered with a Heat Shock Construct Can Rescue Missing Bristles

The results presented above suggest that the formation and maintenance of bristle precursors is a critical period and, that, in the female hybrids it is sensitive to cold temperatures. Maintenance of the precursor fate requires very high levels of Ac-Sc, as well as expression of *asense* (*ase*) (Culi and Modolell, 1998), so the loss of bristles may be due to insufficient levels of Ac-Sc at this time. We therefore looked to see if bristle loss could be alleviated in female hybrids by supplying additional Sc protein by means of a transgenic heat shock construct brought in from the *melanogaster* parent. Animals grown at 18°C were heat shocked for 3 h between 12 and 24 h BPF. There is a significant recovery of bristles (a total loss of 23% bristles in experimental flies compared to 38% in controls, $P < 0.001$, $n = 200$ heminota). All bristle types showed some rescue from this treatment though to varying extents.

We also looked at the effects of heat shock at different times before or after pupariation. The best rescue was obtained from heat shocks applied close to the time of formation of the precursors for each bristle. For example,

the APA and PSC precursors form early between 24 and 12 h before pupariation. These bristles showed some rescue from early heat shocks but a much greater rescue from heat shocks given 6–12 h before pupariation (loss of 41.0 ± 1.29 and 39.00 ± 3.00% after early heat shock, cf. 22.41 ± 1.07 and 16.38 ± 2.81 after late heat shock). They were not rescued by heat shock after pupariation. In contrast the precursor for the PPA bristle forms later, just after pupariation. This bristle is rescued after heat shock at all stages before pupariation but the greatest rescue is seen at pupariation itself and some rescue is still possible after pupariation (39.00 ± 3.42% cf. 22.06 ± 2.21 and 28.75 ± 8.77% APF). We conclude that an exogenous supply of Sc can effect some rescue of the bristles in the hybrid.

Loss of Bristles Is Accentuated by Reducing the Dosage of Achaete–Scute

The preceding results suggest that loss of bristles in the hybrids may be a consequence of reduced levels of Ac-Sc. To investigate further the relationship between bristle loss and levels of *ac-sc* expression, we examined the effects of increasing or decreasing the dosage of *ac-sc* in female hybrids. Various rearrangements of the AS-C were crossed in from the *melanogaster* female parent. Unlike the preceding crosses in which the same two inbred strains were employed in each case, these experiments required the use of a number of different strains with different genetic backgrounds. Therefore, although all other parameters were controlled using the same protocol as before, more variability in the results was encountered. To control for these effects, the percentage of bristle loss in the experimental animals was compared to that of Balancer or other sibling segregants from the same cross (see Materials and Methods).

The AS-C comprises four genes, of which *ac* and *sc* are required for PNC development and *ac*, *sc*, and *ase* are required for precursor development, as well as a number of *cis*-regulatory enhancer elements scattered throughout the 100 kb or so of the complex (Garcia-Bellido, 1979; Ruiz-Gomez and Modolell, 1987; Ghysen and Dambly-Chaudière, 1988; Gomez-Skarmeta *et al.*, 1995). We first employed two deletions, *Df(1)260-1* and $Df(1)sc^{B57}$, that remove the entire AS-C and cause a complete loss of thoracic bristles in patches of homozygous mutant cells in *melanogaster;* when heterozygous, they are without effect (Garcia-Bellido, 1979; Garcia-Bellido and Santamaria, 1977; Gonzalez *et al.*, 1989). The resulting hybrids therefore have only one copy of the AS-C (the *simulans* complex). The results were similar for both deletions and are shown for $Df(1)sc^{B57}$ in Fig. 6A. Loss of all bristles was considerably greater in the experimental females relative to the controls. We conclude that lowering the level of *ac-sc* expression exacerbates bristle loss in the hybrids.

The greater loss of bristles in the deletion cross could be due merely to the lower quantity of Ac-Sc synthesized from the single copy of the AS-C. If this is the only explanation then an additional copy of the AS-C may be expected to alleviate bristle loss. The effect of a duplication of the *melanogaster* AS-C, $Dp(1;2)sc^{19}$, was therefore tested. The results, shown in Fig. 6D, reveal no significant difference in the loss of bristles in female hybrids carrying the duplication. Adding an extra copy of the AS-C from only one of the parental species therefore does not alleviate the phenotype suggesting that it may not lead to a significant increase in the levels of Ac-Sc. Therefore, the increased bristle loss seen in the deletion crosses may not merely be a consequence of having only a single dose of the locus, but also due to the fact that the remaining copy contains exclusively *simulans* sequences.

The $In(1)ac^3\ sc^{10\text{-}1}$ chromosome carries $In(1)ac^3$ that has a breakpoint close to the *ac* promoter and greatly reduced transcription of *ac*, together with $sc^{10\text{-}1}$, a point mutation in the *sc* gene that results in a nonfunctional *sc* protein (Campuzano *et al.*, 1985; Villares and Cabrera, 1987). This chromosome, when homozygous in *melanogaster*, results in loss of function of both Ac and Sc and consequently a total absence of thoracic bristles, a phenotype identical to the two complete deletions (Garcia-Bellido, 1979; Garcia-Bellido and Santamaria, 1977). The pattern of bristle loss in the hybrids heterozygous for this rearrangement is however different. At 25°C two bristles (ASA and APA) did not show a greater frequency of loss than the controls and bristle loss was not quite as extreme as that seen for the deletion crosses (Fig. 6C).

Results described earlier demonstrated the existence of two critical periods concerning the development of bristles in the hybrids, one corresponding roughly to the development of the prepattern for the proneural clusters and the other to formation of the bristle precursors and maintenance of their fate. Both processes require the activity of AS-C *cis*-regulatory sequences and high levels of *ac-sc* expression (Cubas *et al.*, 1991; Skeath and Carroll, 1991; Culi and Modolell, 1998). Hybrids grown at 25°C are sensitized to the first process whereas those grown at 18°C are sensitized to the second. The crosses involving AS-C rearrangements were performed at both 18 and 25°C so that effects specific to either of the two critical periods could be detected. For hybrids bearing a deletion of the AS-C, bristle loss is always greater at 18°C than at 25°C demonstrating that these animals remain cold-sensitive (shown in Figs. 6A and 6B for $Df(1)sc^{B57}$). However, with only one exception (APA), the accentuation of bristle loss in hybrids carrying $Df(1)sc^{B57}$, when compared to control flies, was greater at 25°C than at 18°C (see Fig. 6 and legend). This suggests that flies of this genotype are also very sensitive to the first critical period.

The results observed with $In(1)ac^3\ sc^{10\text{-}1}$, a mutant chromosome that results in absence of Ac and nonfunctional Sc, are somewhat different. At 18°C loss of bristles is unchanged with respect to the sibling controls (not shown).

The fact that bristle loss does not increase at lower temperature suggests that in these animals maintenance of precursor cell fate does not become worse. This may be attributable to the fact that the gene *ase*, whose expression is restricted to the bristle precursors, is still present on this chromosome. At 25°C, on the other hand, bristle loss is greater than it is in controls suggesting that these animals remain sensitive to the first critical period (Fig. 6C). Nevertheless, the effect is not as extreme as that seen for the deletions. The $In(1)ac^{3}$ $sc^{10\text{-}1}$ chromosome differs from those carrying the deletions in that, with the exception of the enhancer element for the ADC and PDC (see below), the *cis*-regulatory elements for PNC formation are not missing. The HSP correlates with expression of the prepattern genes whose products activate *ac*-*sc* expression in the PNCs by means of these position-specific *cis*-regulatory elements. Therefore, a reduction in the level of *ac* and *sc* proteins, but not in the number of copies of most of the AS-C *cis*-regulatory elements, has a less dramatic effect than that caused by a reduction of either the regulatory sequences alone or both regulatory and coding sequences together. We conclude that the greatly increased bristle loss seen in hybrids heterozygous for the two deletions can be attributed not only to a loss of one copy of the *ac* and *sc* coding sequences but also to a loss of the *melanogaster cis*-regulatory elements.

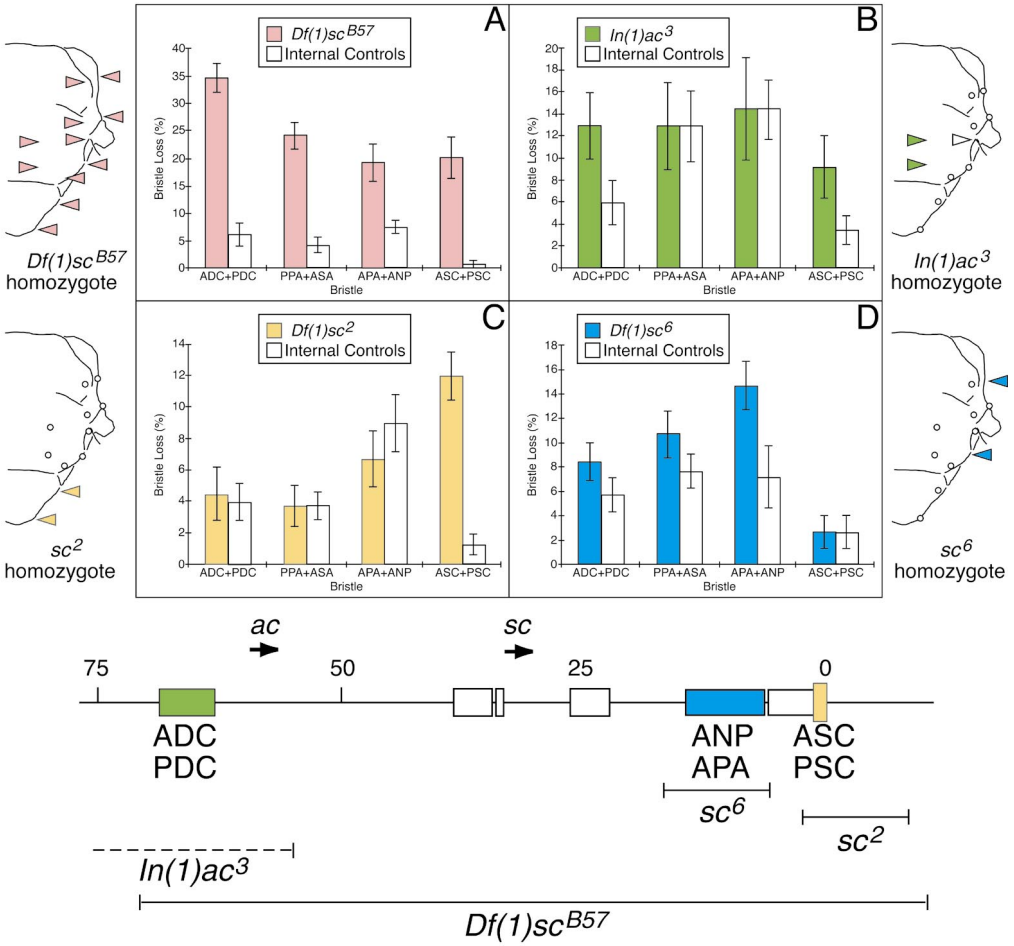
This is consistent with results obtained using sc^{M6}, the only known point mutation in the *melanogaster* AS-C. sc^{M6} corresponds to a nonsense mutation in the *sc* coding sequence; the regulatory regions are unchanged (Gomez-Skarmeta *et al.*, 1995). In *melanogaster* flies hemizygous for sc^{M6}, the PDC, PNP, and PSC bristles are absent; the APA and ASC bristles are lost at high frequency; and the ASA is lost at a lower frequency (Gomez-Skarmeta *et al.*, 1995). Hybrids heterozygous for this mutation, however, display no detectable difference in bristle loss from the controls at 18°C suggesting that here too, maintenance of precursor cell fate is not further impaired (not shown). At 25°C there is a small general low level increase in bristle loss (15.4 ± 1.17 cf. 7.9 ± 0.67 in the controls, $P < 0.001$).

Removal of Specific AS-C Enhancer Elements of D. melanogaster Increases Loss of the Corresponding Bristles in the Hybrid

The enhancer sequences responsible for *ac*-*sc* expression in PNCs at sites corresponding to the formation of some of the bristles have been mapped (Gomez-Skarmeta *et al.*, 1995). We next looked at the effects of removing some of these position-specific elements, either through rearrangements or through small deletions of the *melanogaster* AS-C. We have examined regulatory regions corresponding to four pairs of bristles, the ADC and PDC, the PPA and ASA, the ANP and APA, and the ASC and PSC. The frequency of loss of these bristles from several different crosses grown at 25°C are shown in Fig. 7. In spite of the inherent variability due to the different genetic backgrounds of the parental strains, specific differences between these crosses are consistent with the genetic nature of the rearrangements employed.

FIG. 7. Frequency of loss of bristles in female *melanogaster*/*simulans* hybrids heterozygous for rearrangements of the *achaete–scute* complex, inherited from the *melanogaster* parent, that delete specific *cis*-regulatory sequences. The loss of each bristle is given as a percentage from the 200 or more heminota that were scored for each cross (see Materials and Methods). Sibling flies carrying the *melanogaster* Balancer chromosome were used as controls (see Materials and Methods). Four pairs of bristles were examined. All four have accentuated frequencies of loss in hybrids heterozygous for the complete deletion $Df(1)sc^{B57}$ (A). In contrast only the ADC/PDC, the ASC/PSC, or the APA/ANP pair is lost at higher frequency in animals heterozygous for $In(1)ac^{3}$ (B), $Df(1)sc^{2}$ (C), or $Df(1)sc^{6}$ (D), mutations that remove the *cis*-regulatory sequences for these bristles, respectively ($P < 0.05$ for each case). In the case of $In(1)ac^{3}$ loss of the ASC/PSC pair is also increased ($P < 0.01$). The rearrangements are shown at the bottom and the phenotype of *melanogaster* flies (or mutant patches) homozygous for these mutations is indicated beside each histogram.

FIG. 8. Frequency of bristle loss in female *melanogaster*/*simulans* hybrids heterozygous for deletions of *pannier* or *iroquois*, regulatory genes of *achaete* and *scute*, inherited from the *melanogaster* parent. The domains of expression of these genes, in *melanogaster*, on the adult thorax (extrapolated from the imaginal disc) are shown to the right and the *pnr*-sensitive and *iro*-sensitive bristles are shaded on the histograms. The loss of each bristle is given as a percentage from the 200 or more heminota that were scored for each cross (see Materials and Methods). Sibling flies carrying the *melanogaster* Balancer chromosomes used for these crosses were not viable. Therefore the data from Ore-R crosses have been included for comparison in (A), (C), and (D) (see Materials and Methods). Hybrids doubly heterozygous for $Df(1)sc^{B57}$ and pnr^{VX6} were compared with those heterozygous for $Df(1)sc^{B57}$ alone (B). Hybrids heterozygous for pnr^{VX6} at 25°C (A) display a higher loss of *pnr*-sensitive bristles ($P < 0.05$ for all bristles except the ASC and ADC ($P = 0.71$ and 0.18, respectively)). Hybrids doubly heterozygous for pnr^{VX6} and $Df(1)sc^{B57}$ (B) show a more dramatic loss of the *pnr*-sensitive bristles ($P < 0.05$ for all bristles except the PDC and PPA ($P = 0.072$ and 0.94, respectively). Note that an apparent rescue of some *pnr*-insensitive bristles is seen; it remains unexplained. Similarly bristle loss in hybrids heterozygous for iro^{2} grown at 25°C (C) is dramatically increased for all bristles except the *iro*-insensitive ASC and PSC ($P < 0.01$ in all cases). This includes the ADC and PDC bristles which we have included in the *iro*-sensitive ones in spite of the fact that only the ADC is sometimes missing in certain allelic combinations of *iro* alleles (Leyns *et al.*, 1996). At 18°C, however, only the PSA and ASA display increased loss from this cross (D) ($P < 0.05$).



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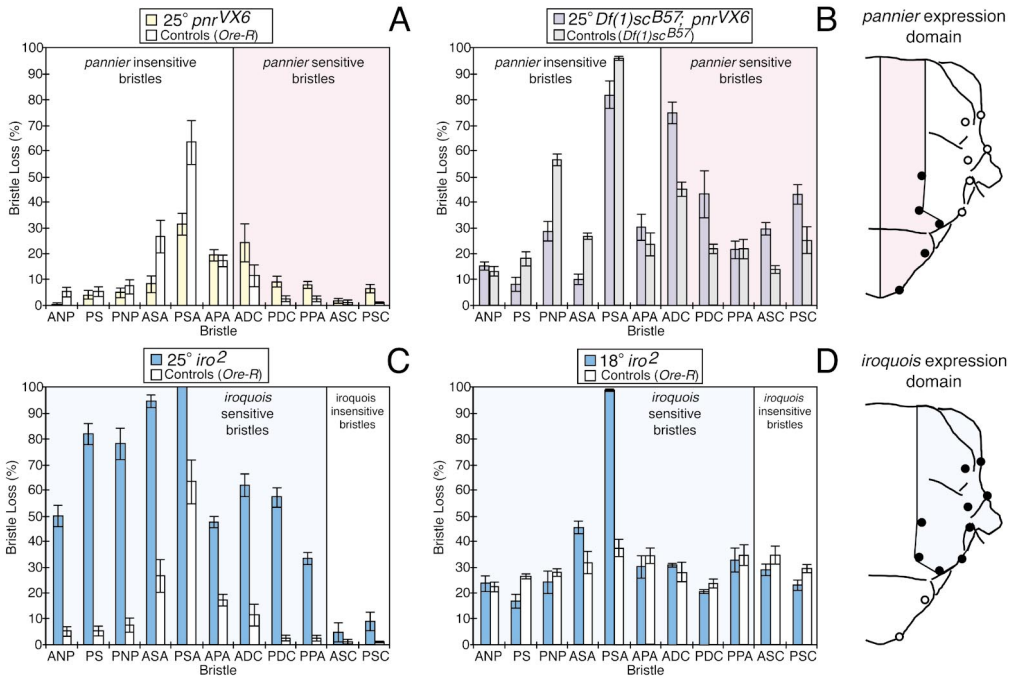

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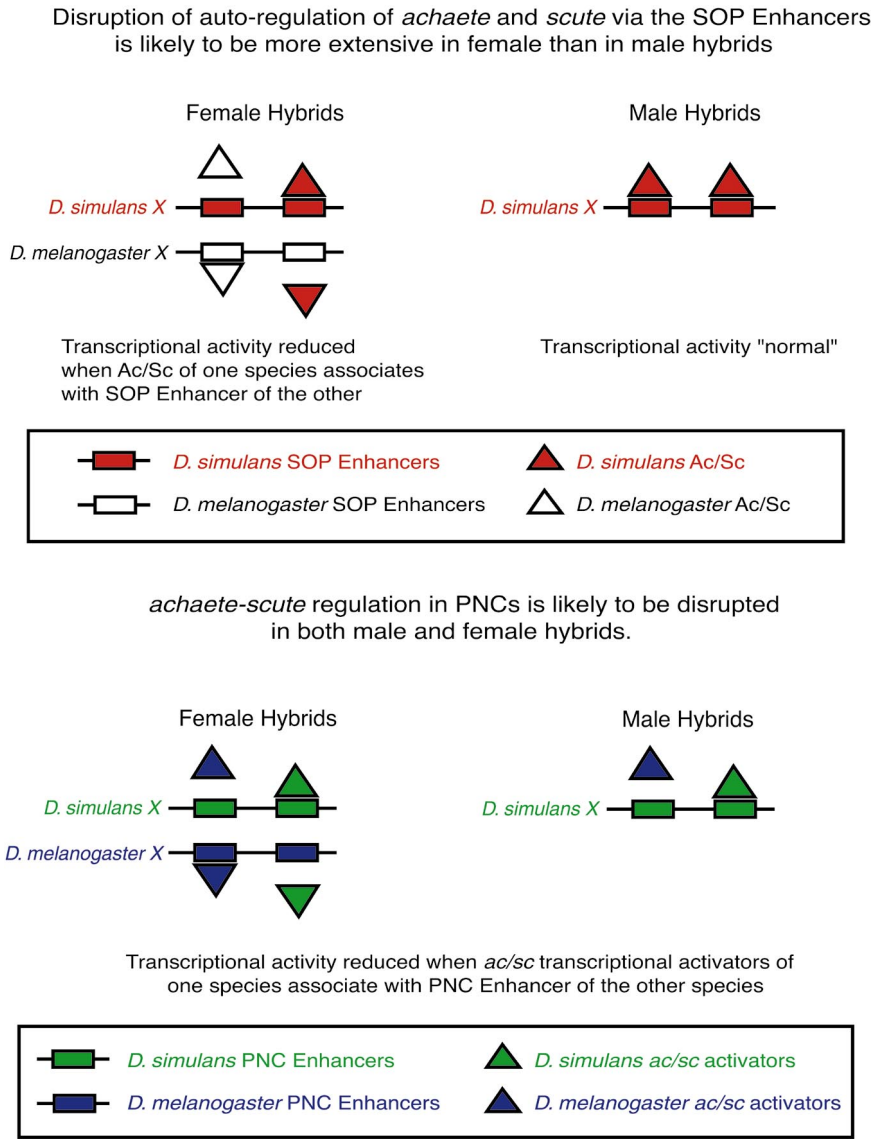

FIG. 9. Model indicating the mechanism proposed to explain the loss of bristles in *melanogaster*/*simulans* hybrids. The hypothesis is that transcriptional activators encoded by the *melanogaster* genome bind less well to, or activate transcription less efficiently from, the regulatory elements of the *simulans achaete–scute* genes and vice versa. Thus, activation in the proneural clusters (PNC) via the PNC enhancer sequences will be impaired in hybrids of both sexes since most of the genes encoding transactivating proteins are autosomal. Autoregulation of *achaete–scute* via the sensory organ precursor enhancers (SOP) and Achaete (Ac) and Scute (Sc) will be less problematic in males with a single X-linked *achaete–scute* locus, since both activators and target regulatory sequences are from the same species.

In hybrids heterozygous for either of the two complete AS-C deletions, all four pairs of bristles are not only frequently missing but are equally affected (Fig. 7A). The AS-C breakpoint of $In(1)ac^3$ separates those regulatory sequences located upstream of the *ac* coding region from the rest of the complex (Gomez-Skarmeta *et al.*, 1995). These include the enhancer for the ADC and PDC bristles. *D. melanogaster* flies homozygous for this inversion are lacking the ADC and PDC (and PSA) bristles (Fig. 7B). Notably the ADC and PDC bristles are lost at a very high frequency in hybrids heterozygous for $In(1)ac^3$ (Fig. 7B). In addition loss of the ASC–PSC pair is also increased, perhaps because of the almost complete loss of the *ac* protein. A significant effect on the ADC–PDC bristles can be seen

therefore in animals heterozygous for a rearrangement in which the enhancer element for these bristles has been moved to a distant location. This effect is only seen under the sensitized conditions that prevail at 25°C; at 18°C bristle loss is not increased (not shown).

sc^6 is an intercalary deletion that removes the regulatory sequences for the ANP and APA bristles, but does not affect any coding sequences (Ruiz-Gomez and Modolell, 1987; Gomez-Skarmeta *et al.,* 1995). *D. melanogaster* flies homozygous for this deletion are lacking the APA bristle but also have reduced *ac-sc* expression in the PNCs corresponding to the APA and ANP bristles (Cubas *et al.,* 1991). In hybrids heterozygous for this deletion at 25°C, the APA-ANP pair of bristles is lost at a much greater frequency than in controls (Fig. 7D). The other three pairs are unaffected. At 18°C in contrast, bristle loss is not increased in experimental compared to control flies (not shown). Thus here too, under sensitized temperature conditions, those bristles dependent upon a specific enhancer element of which only the *simulans* copy is present are missing at a greater frequency than the others.

sc^2, also called ase^1, is an intercalary deletion that removes the regulatory sequences for the ASC and PSC bristles and also the coding sequence of the *ase* gene (Gonzalez *et al.,* 1989). At 25°C, in hybrids heterozygous for this mutation, the loss of the ASC–PSC bristle pair is much greater than that of the other three pairs (Fig. 7C). Loss of the ASC–PSC bristles, but in addition the ADC–PDC pair, is increased at 18°C (not shown). At 18°C the hybrids are weakened for the process of maintenance of precursor cell fate, an effect that might be accentuated in this genotype due to the absence of *ase,* an AS-C gene only expressed in the bristle precursors (Gonzalez *et al.,* 1989).

From the above results we conclude that, in the hybrid, loss of position-specific AS-C enhancer sequences from the *melanogaster* parent is sufficient to cause increased loss of the corresponding bristles. This effect is detected at 25°C, a temperature at which the hybrids are sensitized to processes requiring the activity of these elements.

Removal of D. melanogaster Pannier and Iroquois, Genes Encoding Transcriptional Regulators of Achaete and Scute, Increases Loss of Specific Bristles in the Hybrid

In *melanogaster,* Pannier (Pnr) and three related proteins, Araucan, Caupolican, and Mirror, from the *iroquois (iro)* complex have been shown to act as transcriptional activators of *ac-sc* (Haenlin *et al.,* 1997; Gomez-Skarmeta *et al.,* 1996; Kehl *et al.,* 1998; Garcia-Garcia *et al.,* 1999). *pannier* and *iro* are expressed in broad domains in the thoracic disc, *pnr* is restricted to the medial half, and *iro* is restricted to the lateral half (Fig. 8). Loss of function of these genes causes a loss of those bristles that normally form within their respective domains of expression (Leyns *et al.,* 1996; Ramain *et al.,* 1993; Heitzler *et al.,* 1996; Haenlin *et al.,* 1997). At 25°C hybrids lacking the *melanogaster pnr* gene display increased loss of the PSC, PDC, ADC, and PPA, bristles all known to be affected in *pnr* mutants (Fig. 8A). Loss of these bristles is also greater at 18°C but the effect is not as extreme as that seen at 25°C (not shown). At 25°C the loss of bristles in hybrids lacking the *melanogaster iro* genes is dramatically increased. The ANP, PS, PNP, APA, PDC, ADC, and PPA, all bristles known to be affected in *iro* mutants, are lost at a much greater frequency (Dambly-Chaudière and Leyns, 1992; Leyns *et al.,* 1996; Fig. 8C). Interestingly flies of this genotype are very little affected at 18°C where only the PSA and ASA show an increased loss (Fig. 8D). Therefore, under temperature conditions that weaken the processes leading to expression of the PNCs, hybrids bearing only the *simulans* copy of pnr^+ and iro^+ tend to lose those bristles whose PNCs depend upon activation of *ac-sc* by the products of these genes.

We also examined the effects of hybrids carrying simultaneously a deletion for the AS-C as well as *pnr.* When compared to flies carrying the deletion alone, the *pnr*-dependent bristles, the PSC, ASC, PDC, and ADC, are lost far more frequently than in heterozygotes for the AS-C or *pnr* alone (Fig. 8B). Loss of the other bristles is similar to that seen for the deletion alone.

DISCUSSION

Loss of Bristles in the Hybrids Results from a Reduction in the Levels of Transcription of Achaete and Scute

A number of observations are consistent with the conclusion that the levels of Ac-Sc in the hybrid may be insufficient to sustain bristle development. First, there are two critical periods for bristle development in the hybrids, both revealed through temperature sensitivity. An early heat-sensitive period is correlated with the time when the prepattern of genes whose products activate *ac-sc* in the PNCs is being established. A late cold-sensitive period is correlated with the time at which the bristle precursors form and initiate neural development. Second, an exogenous supply of Ac and Sc, given by *HSSC-2,* can partially alleviate bristle loss. It is most effective when provided during the period at which the PNCs and then the precursors are forming. Third, bristle loss is increased when dosage of the AS-C is reduced. Fourth, the loss of bristles is exacerbated when dosage of the genes encoding upstream activators of *ac* and *sc* is reduced. Fifth, neural development is initiated in the bristle precursors which start to express *neuralized,* but is not maintained, a process known to require high levels of Ac-Sc (Culi and Modolell, 1998). Finally, it was recently demonstrated that the *simulans* X chromosome is the most important genetic component involved in bristle loss (Takano, 1998a). Notably the AS-C is located on the X chromosome.

The loss of bristles in the hybrids thus appears to be due to lowered levels of Ac-Sc function. One possibility is that the Ac-Sc proteins have diverged slightly between *melanogaster* and *simulans* and that as a result they function less effectively on target sequences of the other species. Recent studies have detected 19 fixed nucleotide changes between *melanogaster* and *simulans* in the coding region of *ac* (15 of which are silent; the 4 replacement differences are all conservative amino acid changes) and 26 differences in the *ase* gene, whereas intraspecific variation at the AS-C is low (Eanes *et al.*, 1989; Aguadé *et al.*, 1989; Begun and Aquadro, 1991; Hilton *et al.*, 1994; Akashi, 1996; Takano, 1998b). While it remains probable that the transcriptional efficiency of the proteins has diverged slightly, our observations suggest that this alone cannot account for the loss of so many bristles. Instead, the results are more consistent with the hypothesis that the levels of transcription of the *ac-sc* genes are reduced in the hybrid. For instance, heterozygosity for a nonsense mutation in the *melanogaster sc* coding region has only a very slight effect on bristle loss, suggesting that this is compensated for through regulation of *simulans sc* and the *ac* alleles. Furthermore, while bristle loss can be rescued to some extent by an exogenous supply of *sc* protein, the addition of an extra copy of the *melanogaster* AS-C is without effect. Finally an observation made by Takano (1998a), that removal of one copy of *extramacrochaetae*, a gene encoding a posttranslational repressor of Ac-Sc (Garell and Modolell, 1990; Ellis *et al.*, 1990), is without effect on bristle loss, also suggests that the main defect is upstream of protein function.

Changes in levels of transcription could be attributable to changes in the sequence of the AS-C *cis*-regulatory elements or in their upstream regulatory proteins or both. During development of the bristles, transcription of *ac-sc* is controlled differently at two distinct time periods. The first corresponds to the initial activation of these genes in the PNCs. This is a dynamic process that takes place over a period of 2 days. It relies on a spatial mosaic of *trans*-acting factors and specific *cis*-regulatory elements that regulate transcription at specific sites in the epithelium (Gomez-Skarmeta *et al.*, 1995). In *melanogaster*, each PNC arises within a particular time window, gives rise to one or two bristle precursors, and then regresses (Cubas *et al.*, 1991; Skeath and Carroll, 1991; Cubas and Modolell, 1992). The precursors can be detected early since they continue to express very high levels of protein. At a second time period, the maintenance of high levels of Ac-Sc in the bristle precursors relies on direct autoregulation and crossregulation between *ac* and *sc* (Martinez and Modolell, 1991; Culi and Modolell, 1998). This is achieved through the use of specific enhancer sequences located close to the promoters of *sc*, *ase*, and probably also *ac* (Culi and Modolell, 1998). The sensory organ precursor (SOP) enhancer of *sc* contains the target E box sequence to which Ac and Sc bind (Culi and Modolell, 1998). Therefore, both of these transcriptional events are regulated through *cis*-regulatory sequences scattered throughout the AS-C. Notably, bristle loss in the hybrids is temperature dependent, and the two periods of temperature sensitivity correspond to the developmental intervals at which these two sets of enhancer elements would be required.

The cis-Regulatory Sequences Necessary for Activation of Achaete and Scute in the Proneural Clusters May Have Diverged between the Two Species

Partial deletions of the *melanogaster* AS-C that remove specific enhancer elements required for *ac-sc* expression at particular sites cause increased loss of the corresponding bristles when crossed into the hybrids. Such females are left with only the *simulans* copy of the enhancers. They do, however, have two sets of the transcriptional regulators of *ac-sc*, one from each parent species. One possibility, therefore, is that the transcriptional activators encoded by the *melanogaster* genome bind less well to, or activate transcription less efficiently from, the regulatory elements of the *simulans ac-sc* genes. We thus postulate that in the hybrids transcription of *ac-sc* through the various enhancer elements is reduced when *melanogaster trans*-regulators bind to *simulans* enhancers and vice versa (Fig. 9). This would result in decreased Ac-Sc levels in the PNCs. This hypothesis is also consistent with the observation that removal of the *melanogaster* copy of either *pnr* or *iro*, two loci encoding activators of *ac-sc*, also results in an increased loss of those bristles with PNCs known to require the products of these genes. On the other hand, a duplication of the *melanogaster* AS-C alone, may fail to rescue bristle loss because the proportions of *melanogaster* and *simulans* regulators remain unchanged, and so the levels of transcription may not increase significantly.

It is noteworthy that loss of the *ac-sc* coding sequences, but not of the *cis*-regulatory sequences, from the *melanogaster* parent, as in hybrids heterozygous for $In(1)ac^{3}$ $sc^{10\text{-}1}$ or sc^{M6}, has little effect. Perhaps this may be due to *trans* effects whereby the enhancer sequences on the *melanogaster* chromosome are able to activate transcription from the *simulans* coding sequence. Transvection has recently been described at the very closely linked *yellow* locus (Morris *et al.*, 1999). Many of the enhancer elements are quite far removed from the coding sequences, which presumably means there must anyway be some local looping of the chromatin. It is also possible that the regulatory sequences may titrate the upstream regulatory proteins by binding to them, but this does not account so well for the observed effects on bristle loss.

Activation of transcription in the PNCs appears to be less effective at high temperatures in the hybrids. Loss of

bristles is greater when the larval period is spent at 25°C rather than at 18°C. In female hybrids two bristles (PSA and ASA) display a broad early HSP that correlates with early larval development and the time during which the processes leading to expression of *ac-sc* in the PNCs are initiated. Six other bristles also show signs of heat sensitivity during this same time period. In the male hybrids all bristles are heat-sensitive and the heat-sensitive period is similarly restricted to the early larval stages. The fact that it is the events leading to activation of *ac-sc* in the PNCs that is heat-sensitive is also suggested by the observation that the increased bristle loss due to removal of the *melanogaster* copy of specific enhancer elements is accentuated at 25°C but not at 18°C. Removal of the *melanogaster* copy of the transacting genes *pnr* and, particularly, *iro* also accentuates loss of the corresponding bristles to a greater extent at 25°C than at 18°C.

The heat sensitivity of bristle loss differs between male and female hybrids. This may be attributable to the fact that males have only a single copy of the AS-C. Many of the known genes encoding *trans*-acting factors of *ac-sc* are autosomal. Thus, while both males and females will have equal amounts of the upstream transcriptional activators of *ac-sc* (from both *melanogaster* and *simulans*), the males will only have the *simulans* target AS-C sequences. So if the transcriptional activators encoded by the *melanogaster* genome function slightly less efficiently on the regulatory elements of the *simulans ac-sc* genes, then levels of transcription in the males would be reduced (Fig. 9). The same difficulty will also exist in the females of course, for both *melanogaster* regulators on *simulans* targets and vice versa, but would be slightly less critical since both *melanogaster* and *simulans* target sequences are present (Fig. 9). This may explain the greater heat sensitivity in the males. A single X-linked gene, *Bar,* has been shown to encode a regulator of *ac-sc* (Sato *et al.*, 1999). It has a restricted domain of expression and only affects the PS bristle. Notably this bristle displays the lowest frequency of loss in the male hybrids.

The cis-Regulatory Sequences Necessary for Maintenance of Achaete and Scute Expression in the Precursors May Have Diverged between the Two Species

Our observations, as well as those of Takano (1998a), show that the bristle precursors form but then appear to regress. This is seen by the expression of *neuralized* that is initiated but later lost. Huang *et al.* (1991) observed that, in wild-type animals, initially two or three labeled cells may appear at the site of a sensory organ precursor but that staining is later reduced to a single one. This demonstrates that initiation of expression of *neuralized* precedes a stable commitment to the precursor fate and suggests that in the hybrids the neural fate is not maintained. As described above, maintenance of the precursor fate is known to require high levels of Ac-Sc for some time after the precursor cell has been singled out from the PNC. High levels are sustained due to direct autoregulation via the SOP enhancer sequences (Martinez and Modolell, 1991; Culi and Modolell, 1998). A failure of autoregulation results in a gradual cessation of *ac-sc* expression. This is what appears to happen in the hybrids.

In the female hybrids many of the bristles display cold sensitivity. The CSP is situated precisely at and just after the time of precursor formation. The bristle loss in male hybrids is not cold-sensitive. This suggests that males suffer less from problems in maintaining precursor fate than do females. Again this may be attributed to the presence of a single X chromosome and a single copy of the AS-C, that of *simulans.* If a divergence between SOP enhancer sequences of the two species is responsible for a partial failure of autoregulation in the females, this process would be less critical in the males, as the Ac-Sc proteins and the target SOP enhancer sequences are both from the same species (Fig. 9).

Females heterozygous for a deletion of the *melanogaster* AS-C, would also carry only *simulans* copies of both Ac-Sc and the SOP enhancer. Indeed the cold sensitivity of the bristles in these animals is reduced compared to the controls. Many bristles are still lost at high frequency at 18°C however. This may be due to the fact that, in the females, unlike the males, there will be no dosage compensation for the single copy of the AS-C, so the levels of *ac-sc* expression will still remain lower. In females heterozygous for $In(1)ac^{3}$ $sc^{10\text{-}1}$, which have little or no *melanogaster* Ac and Sc, the bristles are not cold-sensitive.

The Order of Bristle Loss Is Due to Differential Sensitivity to the Two Critical Periods

The relative order of loss of specific bristles differs between male and female hybrids. In light of our hypotheses concerning the nature of the temperature dependence of bristle loss, they would not be expected to be the same. In the males the bristles display a heightened sensitivity to the first event, the setting up of the pattern of proneural clusters. In the females, on the other hand, bristle loss is due to a fragility at both stages, formation of the PNCs and maintenance of precursor fate. Specific bristles appear to differ in their sensitivity to one or both of these processes. It is known that the formation of a bristle precursor is not only dependent upon attaining a critical threshold level of Ac-Sc, but that this varies throughout the disc epithelium such that some areas require much higher levels than others in order to initiate and maintain the precursor cell fate (Campuzano *et al.*, 1986; Balcells *et al.*, 1988; Rodriguez *et al.*, 1990; Cubas and Modolell, 1992). There are thus

spatial differences in competence among the epithelial cells. Furthermore, the PNCs vary both in number of cells and in the duration and the timing of their appearance and each bristle precursor cell arises within a particular time window that may be long or short (Romani *et al.*, 1989; Skeath and Carroll, 1991; Huang *et al.*, 1991; Cubas *et al.*, 1991; Cubas and Modolell, 1992). The order of bristle loss in either sex probably reflects critical threshold levels that differ for each bristle. For example, the PSA is by far the most sensitive bristle in females and it displays both heat- and cold-sensitivity. Its precursor arises from a small cluster of very weak *ac-sc* expression in *melanogaster* and is known to be highly sensitive to perturbation in *ac-sc* expression in this species (Botas *et al.*, 1982; Dambly-Chaudière and Leyns, 1992; Ghysen and Dambly-Chaudière, 1988; Ramain *et al.*, 1993).

We do not know exactly what processes are sensitive to temperature. Both parental species have normal morphology at 18 and 25°C and the effects are unlikely to be due to an inherent temperature sensitivity of the *ac-sc* proteins or their regulators. One possibility, however, is impaired function of protein dimers in which the two protein moieties are not from the same species. This is sometimes observed for animals heterozygous for different mutant alleles of the same gene. The *ac-sc* proteins are known to function as heterodimers together with the product of the *daughterless* gene (Ghysen and Dambly-Chaudière, 1988; Cabrera and Alonso, 1991; Villares and Cabrera, 1987; Gonzalez *et al.*, 1989). The *pnr* protein heterodimerizes with the product of the *u-shaped* gene (Cubbada *et al.*, 1997; Haenlin *et al.*, 1997). The *iro* proteins carry a homeodomain and, although specific partners for Araucan, Caupolican, and Mirror have not been described, cofactors for homeodomain-containing proteins are known (Rauskolb *et al.*, 1993; Mann and Chan, 1996). Perhaps interspecific heterodimers bind to, and activate less effectively, their target sequences. Other aspects of Drosophilid hybrids are also temperature-dependent: viability of *melanogaster–simulans* hybrids is reduced at higher temperature and spermatogenesis in *simulans–mauritiana* hybrids is strongly cold-sensitive (Sturtevant, 1929; Kerkis, 1933; Watanabe *et al.*, 1977; Maside *et al.*, 1998).

CONCLUSIONS

We have confined our analysis to a study of the effects of rearrangements in the AS-C and of two known transcriptional regulators. Clearly many other factors will also impinge upon bristle development and our conclusions do not exclude such effects. From our studies we hypothesize that bristle loss in *melanogaster–simulans* hybrids could be due to slight divergences between the two parental species, in the *cis*-regulatory sequences of the AS-C, in the *ac-sc* proteins themselves, as well as in their transcriptional regulators. This would lead to decreased transcriptional efficiency and an overall reduction in the amount of Ac-Sc in the hybrid. At present, this seems to be the best interpretation for the loss of bristles. Transcriptional impairment would intervene both at the stage of expression in the PNCs and in the autoregulation required for maintenance of precursor fate. Both events rely on regulatory sequences within the AS-C. Other studies have demonstrated that differences in regulatory sequences can be responsible for changes in morphology, or be required to maintain conserved expression domains, between closely related species of the Drosophilidae (Ludwig *et al.*, 1998; Stern, 1998).

One of our observations does not seem to be consistent with this interpretation. Hybrids doubly heterozygous for deletions of *pnr* and the AS-C would only carry the *simulans* copy of both transcriptional regulator and target sequence. This might therefore be expected to alleviate loss of the *pnr*-dependent bristles. Instead these bristles are missing at a much greater frequency than in animals heterozygous for either *pnr* or a deletion of the AS-C alone. A possible interpretation of this is that the gene *pnr*, too, needs to be correctly expressed to wild-type levels in the hybrid. It will also be dependent upon activation by a combination of regulators from both species. If expression of *pnr* is reduced in the hybrid then of course the ability of Pnr to activate *ac-sc* will also be impaired.

Indeed it is likely that the temporal and spatial expression of *ac-sc* at appropriate levels in the PNCs is the result of a cascade of gene activation events that are initiated in early imaginal disc development. As development proceeds, each step of such a sequence may be compromised by partial failure of the earlier ones. In some acute cases failure to achieve a critical threshold level may have an all or nothing effect. This may occur for *ac-sc* expression and bristle precursor development and result in a loss of the precursors. We have found that the rate of development is slightly slower in *simulans* compared to *melanogaster*. This could perhaps cause some asynchrony in the gene activation cascade that would contribute to the incomplete levels of activation of *ac-sc*. Interestingly hybrids between *D. melanogaster* and *D. sechellia* also have missing bristles and take longer to develop than *D. melanogaster* (unpublished observations). Hybrids between *D. mauritiana* and *D. melanogaster*, however, do not have missing bristles in spite of a similarly delayed developmental period (unpublished observations). Thus many steps in the cascade of transcriptional events, starting early in the development of the imaginal discs, might culminate in reduced levels of Ac-Sc in the hybrids.

Changes in the expression patterns of regulatory genes during evolution have generally been studied with respect to small morphological differences between closely related species (see, for example Stern, 1998). Our results suggest an alternative approach that may be equally rewarding. We have looked at the control of gene expression in related

species that display the same morphological pattern but have been separated for a sufficiently long time for the patterning mechanisms to have diverged slightly. The notal bristle pattern within the Drosophilidae has been conserved for at least 50 million years (Grimaldi, 1987). It is presumably under strong selective pressure. This would mean that any small changes in one element of the genetic control would be compensated for by changes in other components. Such a buffering effect could lead, over time, to the concerted evolution of, for example, the coding sequences of the transcriptional regulators and the *cis*-regulatory sequences of the target *ac-sc* genes. Such a phenomenon has been documented in the case of Bicoid and its target *hunchback* between two species of Diptera (Bonneton *et al.*, 1997). Isolation of *ac-sc* regulatory sequences and *pnr* or *iro* coding sequences from *D. simulans* may provide molecular details to support this possibility.

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REFERENCES

Aguadé, M., Miyashita, N., and Langley, C. H. (1989). Reduced variation in the *yellow-achaete-scute* region in natural populations of *Drosophila melanogaster. Genetics* **122,** 607–615.

Akashi, H. (1996). Molecular evolution between *Drosophila melanogaster* and *D. simulans:* Reduced codon bias, faster rates of amino acid substitution, and larger proteins in *D. melanogaster. Genetics* **144,** 1297–1307.

Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M. (1995). Notch signalling. *Science* **268,** 225–232.

Balcells, L., Modolell, J., and Ruiz-Gomez, M. (1988). A unitary basis for different *hairy-wing* mutations of *Drosophila melanogaster. EMBO J.* **7,** 3899–3906.

Begun, D., and Aquadro, C. F. (1991). Molecular population genetics of the distal portion of the X chromosome in *Drosophila:* Evidence for genetic hitchhiking of the *yellow-achaete* region. *Genetics* **129,** 1147–1158.

Biddle, R. L. (1932). The bristles of hybrids between *Drosophila melanogaster* and *Drosophila simulans. Genetics* **17,** 153–174.

Bonneton, F, Shaw, P. J., Fazakerly, C., Shi, M., and Dover, G. A. (1997). Comparison of *bicoid*-dependent regulation of *hunchback* between *Musca domestica* and *Drosophila melanogaster. Mech. Dev.* **66,** 143–156.

Botas, J., Moscoso del Prado, J., and Garcia-Bellido, A. (1982). Gene dose titration analysis in the search for *trans*-regulatory genes in *Drosophila. EMBO J.* **1,** 307–310.

Boulianne, G. L., de la Concha, A., Campos-Ortega, J., Jan L. Y., and Jan, Y. N. (1991). The *Drosophila* neurogenic gene *neuralized* encodes a novel protein and is expressed in precursors of larval and adult neurons. *EMBO J.* **10,** 2975–2983.

Cabrera, C., and Alonso, M. (1991). Transcriptional activation by heterodimers of the *achaete-scute* and *daughterless* gene products of *Drosophila. EMBO J.* **10,** 2965–2973.

Campuzano, S., Balcells, L. I., Villares, R. Carramolino, L., Garcia-Alonso L., and Modolell, J. (1986). Excess function *hairy-wing* mutations caused by *gypsy* and *copia* insertions within structural genes of the *achaete-scute* locus of *Drosophila. Cell* **44,** 303–312.

Campuzano, S., Carramolino, L., Cabrera, C., Ruiz-Gomez, M., Villares, R., Boronat, R., and Modolell, J. (1985). Molecular genetics of the *achaete-scute* complex of *D. melanogaster. Cell* **40,** 327–338.

Cubadda, Y., Heitzler, P., Ray, R., Bourouis, M., Ramain, P., Gelbart, W., Simpson P., and Haenlin M. (1997). *u-shaped* encodes a zinc finger protein that regulates the proneural genes *achaete* and *scute* during formation of bristles in *Drosophila. Genes Dev.* **11,** 3083–3095.

Cubas, P., de Celis, J-F., Campuzano, S., and Modolell, J. (1991). Proneural clusters of *achaete-scute* expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Dev.* **5,** 996–1008.

Cubas, P., and Modolell J. (1992). The *extramacrochaetae* gene provides information for sensory organ patterning. *EMBO J.* **11,** 3385–3393.

Culi, J., and Modolell, J. (1998). Proneural gene self-stimulation in neural precursors: An essential mechanism for sense organ development that is regulated by *Notch* signaling. *Genes Dev.* **12,** 2036–2047.

Dambly-Chaudière, C., and Leyns, L. (1992). The determination of sense organs in *Drosophila:* A search for interacting genes. *Int. J. Dev. Biol.* **36,** 85–91.

Davis, A. W., Roote, J. Morley, T. Sawamura, K., Herrmann, S., and Ashburner, M. (1996). Rescue of hybrid sterility in crosses between *D. melanogaster* and *D. simulans. Nature* **380,** 157–159.

Eanes, W. F., Labate, J., and Ajioka, J. W. (1989). Restriction map variation with the *yellow–achaete–scute* region in five populations of *Drosophila melanogaster. Mol. Biol. Evol.* **6,** 492–502.

Ellis, H. M., Spann D. R., and Posakony, J. W. (1990). *extramacrochaetae,* a negative regulator of sensory organ development in *Drosophila,* defines a new class of helix–loop–helix proteins. *Cell* **61,** 27–38.

Garcia-Bellido, A. (1979). Genetic analysis of the *achaete-scute* system of *Drosophila melanogaster. Genetics* **91,** 491–520.

Garcia-Bellido, A., and Santamaria P. (1977). Developmental analysis of the *achaete–scute* genes of *Drosophila melanogaster. Genetics* **88,** 469–486.

Garcia-Garcia, M-J., Ramain, P., Simpson P., and Modolell, J. (1999). Different contributions of *pannier* and *wingless* to the

patterning of the dorsal *Drosophila* mesothorax. *Development* **126,** 3523–3532.

Garrell, J., and Modolell, J. (1990). The *Drosophila extramacrochaetae* locus, an antagonist of proneural genes that, like these genes, encodes a helix–loop–helix protein. *Cell* **61,** 39–48.

Ghysen, A. (1980). The projection of sensory neurons in the central nervous system of *Drosophila:* Choice of the appropriate pathway. *Dev. Biol.* **78,** 521–541.

Ghysen, A., and Dambly-Chaudière, C. (1988). From DNA to form: the *achaete–scute* complex. *Genes Dev.* **2,** 495–501.

Gomez-Skarmeta, J-L., Diez del Corral, R., de la Calle-Mustienes, E., Ferrés-Marco, D., and Modolell, J. (1996). *araucan* and *caupolican,* two members of the novel *iroquois* complex, encode homeoproteins that control proneural and vein-forming genes. *Cell* **85,** 95–105.

Gomez-Skarmeta, J-L., Rodriguez, I., Martinez, C., Culi, J., Ferrés-Marco, D., Beamonte, D., and Modolell J. (1995). *cis*-regulation of *achaete* and *scute:* Shared enhancer-like elements drive their coexpression in proneural clusters of the imaginal discs. *Genes Dev.* **9,** 1869–1882.

Gonzalez, F., Romani, S., Cubas, P., Modolell J., and Mayor, R. (1989). Molecular analysis of the *asense* gene, a member of the *achaete–scute* complex of *Drosophila melanogaster,* and its novel role in optic lobe development. *EMBO J.* **17,** 181–190.

Grimaldi, D. A. (1987). Amber fossil Drosophilidae (Diptera), with particular reference to the hispaniolan taxa. *Am. Museum Novitates* **2880,** 1–23.

Grimaldi, D. A. (1990). A phylogenetic, revised classification of genera in the Drosophilidae (Diptera). *Bull. Am. Mus. Nat. Hist.* **197.**

Haenlin, M., Cubadda, Y., Blondeau, F., Heitzler, P., Lutz, Y., Simpson P., and Ramain, P. (1997). Transcriptional activity of Pannier is regulated negatively by heterodimerization of the GATA DNA-binding domain with a cofactor encoded by the *u-shaped* gene of *Drosophila. Genes Dev.* **11,** 3096–3108.

Heitzler, P., Haenlin, M., Ramain, P., Calleja M., and Simpson, P. (1996). A genetic analysis of *pannier,* a gene necessary for viability of dorsal tissues and bristle positioning in *Drosophila. Genetics* **143,** 1271–1286.

Hilton, H., Kliman, R. M., and Hey, J. (1994). Using hitchhiking genes to study adaptation and divergence during speciation within the *Drosophila melanogaster* species complex. *Evolution* **48,** 1900–1913.

Huang, F., Dambly-Chaudière, C., and Ghysen, A. (1991). The emergence of sense organs in the wing disc of *Drosophila. Development* **111,** 1087–1095.

Hutter, P. (1997). Genetics of hybrid inviability in *Drosophila. Adv. Genet.* **36,** 157–185.

Kehl, B. T., Cho, K-O., and Choi, K-W. (1998). *mirror,* a *Drosophila* homeobox gene the *iroquois* complex, is required for sensory organ and alula formation. *Development* **125,** 1217–1227.

Kerkis, J. (1933). Einfluss des temperature auf den entwicklung hybriden von *Drosophila melanogaster* x *Drosophila simulans. Wilh. Roux' Arch. Entwicklungsmechanik Organismen* **130,** 1–10.

Kimble, J., and Simpson, P. (1997). The Lin-12/Notch signalling pathway and its regulation. *Annu. Rev. Cell Dev. Biol.* **13,** 333–361.

Lemeunier, F., David, J. R., Tsacas, L., and Ashburner, M. (1984). The *melanogaster* species subgroup. *In* "The Genetics and Biology of *Drosophila*" (M. Ashburner, H. L. Carson, and J. N. Thompson, Eds.), Vol. 3e, pp. 275–294. Academic Press, London.

Leyns, L., Gomez-Skarmeta, J-L., and Dambly-Chaudière, C. (1996). *iroquois,* a prepattern gene that controls the formation of bristles on the thorax of *Drosophila. Mech. Dev.* **59,** 63–72.

Lindsley, D. L., and Zimm, G. (1992). "The Genome of *Drosophila melanogaster.*" Academic Press, San Diego.

Ludwig, M. Z., Patel, N., and Kreitman, M. (1998). Functional analysis of *eve* stripe enhancer evolution in *Drosophila:* Rules governing conservation and change. *Development* **125,** 949–958.

Mann, R. S., and Chan, S-K. (1996). Extra specificity from *extradenticle:* The partnership between hox and pbx/exd proteins. *Trends Genet.* **12,** 258–262.

Martinez, C., and Modolell, J. (1991). Cross regulatory interactions between the proneural *achaete* and *scute* genes of *Drosophila. Science* **251,** 1485–1487.

Maside, X. R., Barral J. P., and Naveira, H. (1998). Hidden effects of X chromosome introgressions on spematogenesis in *Drosophila simulans* × *D. mauritiana* hybrids unveiled by interactions among minor genetic factors. *Genetics* **150,** 745–754.

McAlpine, J. F. (1981). "Manual of Nearctic Diptera" (J. F. McAlpine, Ed.), Monograph No. 28, Vol. 2. Research Branch Agriculture Canada.

Modolell, J., and Campuzano, S. (1998). The *achaete–scute* complex as an integrating device. *Int. J. Dev. Biol.* **42,** 275–282.

Morris, J. R., Chen, J-L., Filandrinos, S. T., Dunn, R. C., Fisk, R., Geyer P. K., and Wu, C-T. (1999). An analysis of transvection at the *yellow* locus of *Drosophila melanogaster. Genetics* **151,** 633–651.

Morrison, A., Hodgetts, C., Gossler, A., Hrabé de Angelis, M., and Lewis, J. (1999). Expression of *Delta1* and *Serrate1 (Jagged1)* in the mouse inner ear. *Mech. Dev.* **84,** 169–172.

Ramain, P., Heitzler, P. Haenlin, M., and Simpson, P. (1993). *pannier,* a negative regulator of *achaete* and *scute* in *Drosophila,* encodes a zinc finger protein with homology to the vertebrate transcription factor GATA-1. *Development* **119,** 1277–1291.

Rauskolb, C., Peifer M., and Weischaus, E. (1993). *extradenticle,* a regulator of homoetic gene activity is a homolog of the homeobox-containing human proto-oncogene *pbx1. Cell* **74,** 1101–1112.

Rodriguez, I., Hernandez, R., Modolell, J., and Ruiz-Gomez, M. (1990).Competence to develop sensory organs is temporally and spatially regulated in *Drosophila* epidermal primordia. *EMBO J.* **9,** 3583–3592.

Romani, S., Campuzano, S., Macagno E., and Modolell, J. (1989). Expression of *achaete* and *scute* genes in *Drosophila* imaginal discs and their function in sensory organ development. *Genes Dev.* **3,** 997–1007.

Ruiz-Gomez, M., and Modolell, J. (1987). Deletion analysis of the *achaete–scute* locus of *Drosophila melanogaster. Genes Dev.* **1,** 1238–1246.

Sato, M., Kojima, T., Michiue, T., and Saigo, K. (1999). *Bar* homeobox genes are latitudinal prepattern genes in the developing *Drosophila* notum whose expression is regulated by the concerted functions of *decapentaplegic* and *wingless. Development* **126,** 1457–1466.

Simpson, P. (1996). *Drosophila* development: A prepattern for sensory bristles. *Curr. Biol.* **6,** 948–950.

Skeath, J., and Carroll, S. B. (1991). Regulation of *achaete–scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. *Genes Dev.* **5,** 984–995.

Stern, C. (1954). Two or three bristles. *Am. Sci.* **42,** 213–247.
Stern, D. (1998). A role of *Utlrabithorax* in morphological differences between *Drosophila* species. *Nature* **396,** 463–466.
Sturtevant, A. H. (1919). A new species closely resembling *Drosophila melanogaster. Psyche* **26,** 153–155.
Sturtevant, A. H. (1920). Genetic studies on *Drosophila simulans. Genetics* **5,** 488–500.
Sturtevant, A. H. (1929). The genetics of *Drosophila simulans. Carnegie Institute of Washington Publ.* **399,** 1–62.
Takano, T. (1998a). Loss of notum macrochaetae as an interspecific hybrid anomaly between *Drosophila melanogaster* and *D. simulans. Genetics* **149,** 1435–1450.
Takano, T. (1998b). Rate variation of DNA sequence evolution in *Drosophila* lineages. *Genetics* **149,** 959–970.
Villares, R., and Cabrera, C. V. (1987). The a*chaete–scute* gene complex of *D. melanogaster:* conserved domains in a subset of genes required for neurogenesis and their homology to *myc*. *Cell* **50,** 415–424.
Watanabe, T. K., Lee, W. H., Inoue, Y., and Kawanishi, M. (1977). Genetic variation of the hybrid crossability between *Drosophila melanogaster* and *D. simulans. Jpn. J. Genet.* **52,** 1–8.

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